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*With amended claims.*

(54) Title: TOXIN GENE FROM XENORHABDUS NEMATOPHILUS

(57) Abstract

Purified insecticidal toxins and biologically active fragments thereof, and polynucleotide molecules encoding same, from the bacteria *Xenorhabdus nematophilus* are described.

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# TOXIN GENE FROM XENORHABDUS NEMATOPHILUS

## Technical Field

The present invention concerns the identification and isolation of a new class of protein toxins specific against insects which are produced by bacteria from the species *Xenorhabdus nematophilus* and possibly by the species *X.beddingii*. In addition, the present invention relates to the insertion of this class of toxin into recombinant viruses, bacteria, protozoa, fungi, and transgenic plants in order to broaden the use of these toxins for control of a large range of insect pests and plant parasitic nematodes.

## Background

Insect pathogenic nematodes of the family *Steinernematidae* are known to be symbiotically associated with bacteria of the genus *Xenorhabdus*. It has been observed that these bacteria have the ability to kill a wide range of different insects without the aid of their nematode partners.

The present inventors have identified a new class of toxins. A DNA fragment encoding one of these toxins has been isolated from *Xenorhabdus nematophilus* stain A24 and characterised by sequencing. As will be recogised by persons skilled in the art, DNA fragments encoding members of this new class of toxins may be usefully introduced into viral agents, including entomopox and nuclear polyhedrosis viruses; bacteria (including *Gracilicutes, Firmicutes, Tenericutes* and *Mendosicutes*); fungi; protozoa; and plants.

## Summary of the Present Invention

In a first aspect, the present invention consists in a polynucleotide molecule comprising a nucleotide sequence which encodes an insecticidal toxin and which is at least 70% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919, or a fragment thereof which fragment encodes an insecticidal polypeptide.

In a preferred embodiment of the present invention the nucleotide sequence is at least 90% to the sequence shown in Table 1 from residue 83 to 919.

Preferably, the nucleotide sequence which encodes an insecticidal toxin from *Xenorhabdus* and more preferably, the nucleotide sequence substantially corresponds to the sequence shown in Table 1 from residue 83 to 919.

In a second aspect the present invention provides in an insecticidal toxin which includes an amino acid sequence which is at least 70% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

In a preferred embodiment of the present invention the insecticidal toxin includes an amino acid sequence which is at least 90% homologous to residues 1 to 278 shown in Table 1 or a functional fragment thereof.

In a further preferred embodiment the insecticidal toxin includes an amino acid sequence substantially corresponding to residues 1 to 278 in Table 1 or a functional fragment thereof.

In a third aspect the present invention provides in a recombinant organism, the organism being characterised in that it is transformed with the polynucleotide molecule of the first aspect of the present invention.

The organisms which may be usefully transformed with the polynucleotide molecule of the first aspect of the present invention include viral agents such as entomopox and nuclear polyhedrosis viruses; bacteria, such as *Gracilicutes, Firmicutes, Tenericutes* and *Mendosicutes;* fungi; protozoa; and plants.

The term "substantially corresponds" as used herein in relation to the nucleotide sequence is intended to encompass minor variations in the nucleotide sequence which due to degeneracy do not result in a change in the encoded protein. Further this term is intended to encompass other minor variations in the sequence which may be required to enhance expression in a particular system

but in which the variations do not result in a decrease in biological activity of the encoded protein.

The term "substantially corresponding" is used herein in relation to the amino acid sequence is intended to encompass minor variations in the amino acid sequence which do not result in a decrease in biological activity of the insecticidal toxin. These variations may include conservative amino acid substitutions. The substitutions envisaged are:-

G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkalamino acids.

As used herein the term "functional fragments" is intended to encompass fragments of the insecticidal toxin which retain insecticidal activity.

In a fourth aspect, the present invention provides a method for controlling the proliferation of insects, comprising applying to an infested area a recombinant organism according to the third aspect optionally in admixture with an acceptable agricultural carrier.

**Isolation and Characterisation of a Toxin from *Xenorhabdus nematophilus* A24**

**Generation of a Cosmid Library**

Genomic DNA from *Xenorhabdus nematophilus* A24, isolated using the method of Marmur (1961) was partially digested using the restriction enzyme Sau 3A, to generate fragments of DNA that were in the size range of 30 to 50 kilobasepairs (kb), and dephosphorylated using the enzyme calf alkaline phosphatase. The cosmid "Supercos" (Stratagene) was prepared to receive foreign insert DNA into its <u>Bam</u> HI cloning site according to the manufacturer's instructions. The digested DNA from *X.nematophilus* A24 was added to the cosmid DNA in a ratio of 3:1 and ligated together using the enzyme T4 DNA ligase. The ligated material was subsequently packaged into λ-bacteriophage using the Gigapack II XL Packaging Extract (Stratagene) as per the manufacturer's

instructions. The packaged DNA was subsequently transfected into the *Escherichia coli* strain NM554 (F-, recA, araD139,Δ (ara, leu) 7696, Δlac Y74, galU-, galK-, hsr, hsm$^+$, strA, mcrA[-], mcrB[-]. Bacteria were plated out onto Luria Bertani (LB) agar plates containing 150 μg ml$^{-1}$ ampicillin to select for those bacteria containing recombinant Supercos plasmids.

**Screening for Toxin Producing Clones**

Individual clones were grown overnight at 28$^{\circ}$C in LB containing 150 μg ml$^{-1}$ ampicillin. Cultures were treated for 15 minutes with 2mg ml$^{-1}$ lysozyme in order to release any proteins produced by the recombinant DNA into the medium. Five μl aliquots of this solution were then injected directly into the haemocoel of three *Galleria mellonella* fourth instar larvae. Appropriate controls containing lysozyme and non-recombinant *E.coli* NM554 cultures were also injected to confirm the absence of any toxicity to these larvae. Two clones were found to have strong insecticidal activity. Injected larvae were found to be very sluggish after 30 hours, with all larvae dead within three days.

Characterisation of Toxin Producing Clones

The recombinant Supercos DNA from these clones was isolated using an alkaline lysis procedure (Maniatis et al., 1982). Isolated DNA was digested with varying restriction enzymes and analysed using TAE agarose gel electrophoresis (Maniatis et al, 1982). It was found that both clones were identical and contained a 34.6 kb DNA insert from *X. nematophilus* A24. One of these clones cos149 was chosen for further study.

A 7.4kb Bam HI fragment from cos149 was cloned into the plasmid vector pGEM7Z(f)+ (Promega) which was transformed into the *E.coli* strain DH5α (F-, Φ80dlac ZΔ M15, recA1, endA1, gyrA96, thi-1, hsdR17[$r_K$-, $m_K$+] sup E44, relA1, deoR, Δ[lacZYA-argF] U169) using electroporation at 25μF, 200Ω and 2.5kV in a 0.2cm

cuvette in a Bio-Rad Gene Pulser. This clone (N8pGEM) was found to continue to be toxic against *G.mellonella* larvae.

Plasmid DNA from N8pGEM was isolated and digested with the restriction enzymes <u>Cla</u>I and <u>Sph</u>I. This resulted in the linearization of this plasmid containing one end (3') which was resistant to digestion by the enzyme Exonuclease III and the other end (5') which could be digested at a constant rate of 450 bases per minute at 37$^{O}$C by this enzyme using the Erase-a-Base kit from Promega. Using this enzyme aliquots containing decreasing size plasmids were obtained which were recircularised using the enzyme T4 DNA ligase. Recircularised plasmids were reintroduced into the bacterium *E.coli* strain DH5a using electroporation (see above). Varying size clones were selected and used for injecting *G.mellonella* larvae. The smallest clone which continued to be insecticidal was found to contain 1.5kb of *X.nematophilus* A24 DNA and was designated tox 1.

Plasmid DNA from tox 1 was isolated and digested with the restriction enzymes <u>Sac</u> I and <u>Hind</u>III, respectively to again create linear molecules with one end resistant and the other sensitive to digestion with Exonuclease III. Deletion mutants were isolated and tested against *G.mellonella* larvae. A clone which now only contained 1.2kb of *X.nematophilus* A24 DNα was isolated and was toxic against our test insect. This clone was designated toxb4.

The recombinant plasmids from toxb4 and three further (non-toxic) deletion clones, toxb5, toxb6 and toxb7, were isolated and used for obtaining the sequence of both strands of the toxin gene. Sequencing was performed using the Applied Biosystems, Incorporated Model 370 automated sequencer. Sequencing templates were prepared using double stranded DNA templates and the 21M13 and SP6 primer sites located on the pGEM7Z(f)+ plasmid and

using the Taq dye primer cycle sequencing protocol (Applied Biosystems, Incorporated).

The toxin gene was found to consist of an 834 basepair open reading frame (Table 1) which translates into a 278 amino acid protein (Table 2). The start of the toxin gene sequence was preceded by appropriate DNA promoters necessary for transcription of the gene into a mRNA molecule prior to its synthesis into a peptide. These consist of a Shine-Dalgarno poly-purine sequence and -10 and -35 RNA polymerase recognition sequences (Table 1).

The DNA sequence and the derived amino acid sequences were analysed by sequence data bank analyses to determine if any other related sequences have previously been identified. The results indicated that no other sequence exists in the GenBank and EMBL data banks which has any similarity to this gene and its product.

Cloning of Xenorhabdus Toxin into a High-Expression Vector

Using the determined DNA sequence, 20-mer DNA primers were designed to cover the 5′ and 3′ region of the toxin gene and thus allow PCR amplification of the toxin and subsequent insertion into an expression vector. These primers included linker regions containing appropriate restriction enzyme sites (<u>Cla</u>I and <u>Nde</u>I for the 5′ primer and <u>Bam</u> HI for the 3′ primer).

5′ primer CCATCGATCATATGGTTATTAAACC

3′ primer CGGGATCCTTATCTCTAAGGTTTTT

Utilising a standard PCR protocol (Innis, M.A., Gelford, D.H., Sminsky, J.J. and White, T.J.: (1990). PCR Protocols : A Guide to Methods and Applications. Academic Press, San Diego. 482pp) the toxin was amplified out of the genome of *X.nematophilus* A24 and restriction digested with <u>Cla</u> I and <u>Bam</u> HI. The digested fragment was subsequently ligated into pGEM-7Zf(+) and then subcloned from this vector into the high expr ssion vector pT7T2b(derived from pET11 [Novagen] and carrying the T7

promoter upstream from the start of the toxin insert; constructed by Dr. Karl Gordon, CSIRO, Division of Entomology) using the restriction enzyme sites Nde I and Bam HI. The recombinant plasmid was transformed into the E. coli strain BL21(DE3)[F-ompT $r_B$ -$m_B$ -, which carries in its chromosome the T7 RNA polymerase gene under lac UV5 control). Induction of the toxin may be achieved by the addition of 0.4mM IPTG at mid-exponential phase of the culture and continuing the incubation for an extra 4 hours.

*In vitro* expression of the 1.2 Kb insert fragment from toxb4 was achieved with the *E.coli* S30 Extract Procaryotic Translation System for linear DNA. Only a 30kDa peptide was produced indicating that the 1.2 Kb fragment encodes one peptide only - the insect toxin.

**Southern Blot Hybridization of a Range of Xenorhabdus spp. and Photohabdus Luminescens Strains with the X. nematophilus A24 Toxin Gene**

DNA isolated from a range of *Xenorhabdus* species and *Photohabdus* (bacteria symbiotically associated with nematodes from the family *Heterohabditidae*) controls was digested to completion with the restriction enzyme Eco RV and run out on a 0.8% TAE agarose gel and the DNA fragments blotted and fixed onto a Hybond-N+ membrane (Amersham) as per the manufacturer's instructions.

The toxin gene was radiolabelled with $^{32}P$ using nick translation (Maniatis *et al.*, 1982) and probed against the blot containing the DNA of a range of *Xenorhabdus* and *Photohabdus* strains (Maniatis *et al.*, 1982). Under moderate stringency wash conditions at 65°C(0.1% SDS, 1% SSPE, Maniatis *et al*, 1982) the toxin only hybridised to *X. nematophilus* and *X. beddingii* strains. However, the toxin gene did not show any homology to the DNA from strains of *X. bovienii*, *X. poinarii*, some unclassified *Xenorhabdus spp.* and *Photohabdus luminescens*. This result suggests that this toxin type is confined to strains from

the species *X. nematophilus* and *X. beddingii*. As *X. beddingii* has insecticidal activity and shows homology to the toxin gene it is most probable that these sequences are part of related/similar yet slightly different toxins. A high stringency wash at 65°C(0.1% SDS, 0.1% SSPE; Maniatis *et al.* 1982) of the blot removed the message from the *X.beddingii* strain, but not from the *X.nematophilus* strains.

Characteristics of the Toxic Protein Product

The toxin is inactivated by heating to 65°C for 15 minutes, yet stable at 45°C. Sodium dodecyl sulphate at a concentration of 0.1% does not inactivate this toxin thereby indicating extreme stability and thereby a protein which will fold into its appropriate form under a wide range of different conditions (which includes most cell types).

This new class of toxin may be purified by one or more methods of protein purification well known in the art. Insecticidal fragments may be generated from the purified toxin using, for example, cleavage with trypsin or cyanogen bromide.

As will be appreciated by those skilled in this field, the present invention provides a new class of toxins useful for genetically engineering a wide range of biological systems which will thus become more useful for control of insect pests detrimental to agricultural, aquatic and forest industries.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

# TABLE 1

1 AAGAAACCGT AACAGCGGAA ATCAACGCTG CAATTTATAT TAGTAGTCAT

Start -35

51 TTCAATAAAC GCCAACATAA TGGGAAAGTA CAATGGTTAT TAAACCCGTA

-10 S-D

101 ACAACTCCGA GTGTAATACA ATTAACGCCT GATGATAGAG TAACGCCTGA

151 TGATAAAGGT GAATATCAAC CCGTTGAAAA GCAAATAGCG GGAGATATAA

201 TACGTGTACT AGAATTCAAG CAAACAAATG AAAGTCATAC AGGATTGTAT

251 GGAATTCCAT ATCGAGCTAA GAAAGTAATA ATAGCATATG CTTTAGCGGT

301 AAGTGGTATT CATAATGTCT CTCAACTTCC AGAAGACTAT TATAAAAATA

351 AGGATAACAC AGGTAGAATT TATCAAGTAT ACATGTCTAA TCTTTTATCT

401 GCACTATTGG GTGAGAATGG TGATCAAATT TCTAAAGATA TGGCAAATGA

451 TTTTACCCAG AACGAACTGG AGTTTGAGGT CAACGTCTTA AAAATACCTG

501 GGATATTCCT GATCTTGAGA ATAAACTATT GGAAGATTTA TTCAGATGAA

551 GATAAATTAT TAGCACTATA TTTCTTTGCT TCACAAGAAC TTCCAATGGA

601 GGCAAATCAA CAATCAAATG CAGCAAATTT TTTTAAAGTA ATTGATTTTT

651 TACTTATCTT ATCTGCTGTA ACATCACTGG GAAAAAGGAT TTTTTCAAAA

701 AATTTTTACA ATGGTCTAGA AACTAAATCA TTAGAGAATT ATATTGAGAG

751 AAAAAAACTT TCTAAACCTT TCTTTCGACC ACCGCAGAAG TTACCTGATG

801 GCAGAACAGG CTACTTGGCC GGTCCAACAA AAGCGCCTAA ATTGCCAACA

851 ACGTCTTCTA CAGCAACAAC GTCTACAGCA GCTTCATCTA ATTGGAGAGT

901 TAGTTTGCAA AAACCTTAGA GATAACCCAT CCAGAAATAC ATTTATGAAA

Stop

951 ATGGATGATG CTGCAAAACG AAAATATAGT TCATTTATAA AAGAGGTACA

1001 AAAGGGTAAT GATCCACGTG CAGCAGCAGC AAGTATTGGT ACAAAAAGCG

1051 GCAGTAACTT CGAAAAACTG CAAGGTAGAG ATTTATATAG TATAAGACTA

1101 AGCCAAGAAC ACAGGGTAAC ATTCTCCATA AATAATACTG ACCAAATAAT

1151 GGAGATCCAA AGTGTTGGAA CTCATTACCA AAATATATAA CCTGATTTAT

1201 AGTAGTGATA AGACGTAAGA TAAATATGGA AGGTTGTAAT TCTATTGCAC

1251 TTCCTCAGAG GTGACCGCTC AG

TABLE 2

```
  1  MVIKPVTTPS VIQLTPDDRV TPDDKGEYQP VEKQIAGDII RVLEFKQTNE
 51  SHTGLYGIPY RAKKVIIAYA LAVSGIHNVS QLPEDYYKNK DNTGRIYQVY
101  MSNLLSALLG ENGDQISKDM ANDFTQNELE FEVNVLKIPG IFLILRINYW
151  KIYSDEDKLL ALYFFASQEL PMEANQQSNA ANFFKVIDFL LILSAVTSLG
201  KRIFSKNFYN GLETKSLENY IERKKLSKPF FRPPQKLPDG RTGYLAGPTK
251  APKLPTTSST ATTSTAASSN WRVSLQKP*R *PIQKYIYEN G*CCKTKI*F
301  IYKRGTKG** STCSSSKYWY KKRQ*LRKTA R*RFI*YKTK PRTQGNILHK
351  *Y*PNNGDPK CWNSLPKYIT *FIVVIRRKI NMEGCNSIAL PQR*PL
```

CLAIMS:

1. A polynucleotide molecule comprising a nucleotide sequence which encodes an insecticidal toxin and which is at least 70% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919, or a fragment thereof which fragment encodes an insecticidal polypeptide.

2. A polynucleotide molecule as claimed in claim 1 in which the nucleotide sequence is at least 90% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919.

3. A polynucleotide molecule comprising a nucleotide sequence substantially corresponding to the sequence shown in Table 1 from residue 83 to 919 or a fragment thereof, which fragment encodes an insecticidal polypeptide.

4. A polynucleotide molecule according to claim 7, wherein the nucleotide sequence encodes an insecticidal toxin, or an insecticidal fragment thereof, from *Xenorhabdus nematophilus*

5. A polynucleotide nucleotide molecule according to any one of claims 1 to 4 in which the molecule is a DNA molecule.

6. A purified insecticidal toxin, or functional fragment thereof, from the bacterial genus *Xenorhabdus*.

7. A purified insecticidal toxin, or functional fragment thereof, from *Xenorhabdus nematophilus*.

8. An insecticidal toxin which includes an amino acid sequence which is at least 70% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

9. An insecticidal toxin as claimed in claim 8 in which the toxin includes an amino acid sequence which is at least 90% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

10. An insecticidal toxin, the toxin including an amino acid sequence substantially corresponding to residues 1 to 278 shown in Table 1 or a functional fragm nt thereof.

11. A recombinant organism characterised in that it is transformed with the polynucleotide molecule according to any one of claims 1 to 5.

12. A recombinant organism according to claim 10 selected from the group consisting of entomopoxvirus, nuclear polyhedrosis virus, bacteria, fungi, protozoa and plants.

13. A method for controlling the proliferation of insects, comprising applying to an infested area a recombinant organism according to claim 10 or 11 optionally in admixture with an acceptable agricultural carrier.

AMENDED CLAIMS

[received by the International Bureau on 25 November 1994 (25.11.94); original claims 6 and 7 amended; remaining claims unchanged (1 page)]

1. A polynucleotide molecule comprising a nucleotide sequence which encodes an insecticidal toxin and which is at least 70% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919, or a fragment thereof which fragment encodes an insecticidal polypeptide.

2. A polynucleotide molecule as claimed in claim 1 in which the nucleotide sequence is at least 90% homologous to the nucleotide sequence shown in Table 1 from residue 83 to 919.

3. A polynucleotide molecule comprising a nucleotide sequence substantially corresponding to the sequence shown in Table 1 from residue 83 to 919 or a fragment thereof, which fragment encodes an insecticidal polypeptide.

4. A polynucleotide molecule according to claim 7, wherein the nucleotide sequence encodes an insecticidal toxin, or an insecticidal fragment thereof, from *Xenorhabdus nematophilus*

5. A polynucleotide nucleotide molecule according to any one of claims 1 to 4 in which the molecule is a DNA molecule.

6. A purified insecticidal protein, or functional fragment thereof, from the bacterial genus *Xenorhabdus*.

7. A purified insecticidal protein, or functional fragment thereof, from *Xenorhabdus nematophilus*.

8. An insecticidal toxin which includes an amino acid sequence which is at least 70% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

9. An insecticidal toxin as claimed in claim 8 in which the toxin includes an amino acid sequence which is at least 90% homologous to residues 1 to 278 shown in Table 2 or a functional fragment thereof.

10. An insecticidal toxin, the toxin including an amino acid sequence substantially corresponding to residues 1 to 278 shown in Table 1 or a functional fragment th reof.

**A. CLASSIFICATION OF SUBJECT MATTER**

Int. Cl.$^6$ C12N 15/31, C12N 5/10, C12P 21/02, A01N 63/02

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
Derwent Database: file WPAT: Chemical Abstracts Service: file CASM. See "Electronic database" box for keywords.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: C12N 15/31

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
Derwent database, file WPAT; Chemical Abstracts service, file CASM; Keywords: "Xenorhabdus and (nematophilus or beddingii)"; "Akhurst" (in WPAT), "Akhurst and Xenorhab:" in CASM.
STN International, file CA, sequence "CCGTTGAAAAGCAAA" and "PMEANQQSNA".

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to Claim N . |
|---|---|---|
| X | AU,B,21230/83 (558287) (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL ORGANISATION) 22 May 1984 (22.05.84) See entire specification especially page 3 lines 15-29. | 6,7 |
| X | B.V. McINERNEY et al: "Biologically active metabolites from Xenorhabdus spp, Part 1. Dithiolopyrrolone derivatives with antibiotic activity". Journal of Natural Products, Vol. 54, number 3, pp. 774-784, May-June 1991. See abstract, page 779 last 2 paragraphs, page 780 Table 2, page 781 first paragraph | 6,7 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 6 September 1994 (06.09.94) | 30 SEPTEMBER 1994 (30.09.94) |

| Name and mailing address of the ISA/AU | Authorized officer |
|---|---|
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| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category* | Citation of document, with indication, where appropriate of the relevant passages | Relevant t Claim No. |
| X | B.V. McINERNEY et al: "Biologically active metabolites from Xenorhabdus spp, Part 2. Benzopyran-1-one derivatives with gastroprotective activity", Journal of Natural Products, Vol. 54, number 3, pp. 785-795, May-June 1991. | 6,7 |

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Member | | | | | |
|---|---|---|---|---|---|---|---|
| AU | 21230/83 | CA | 1214130 | EP | 126092 | US | 4672130 |
| | | WO | 84/01775 | ZA | 8307974 | | |

**END OF ANNEX**